Chemico-Biological Interactions 206 (2013) 272–278

Contents lists available at ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

Effect of menthol in experimentally induced ulcers: Pathways of gastroprotection

A.L. Rozza [a], C.A. Hiruma-Lima [b], R.K. Takahira [c], C.R. Padovani [d], C.H. Pellizzon [a,*]

[a] Morphology Department, Biosciences Institute, UNESP – Univ Estadual Paulista, Botucatu/SP, Brazil
[b] Physiology Department, Biosciences Institute, UNESP – Univ Estadual Paulista, Botucatu/SP, Brazil
[c] Veterinary Clinics Department, School of Veterinary Medicine and Animal Sciences (FMVZ), UNESP – Univ Estadual Paulista, Botucatu/SP, Brazil
[d] Biostatistics Department, Biosciences Institute, UNESP – Univ Estadual Paulista, Botucatu/SP, Brazil

ARTICLE INFO

Article history:
Received 3 July 2013
Received in revised form 8 August 2013
Accepted 1 October 2013
Available online 9 October 2013

Keywords:
Menthol
Gastroprotection
Gastric ulcer
Diarrhea
Acute toxicity

ABSTRACT

Based on ethnopharmacological indications that Mentha species may be used in the treatment of gastrointestinal diseases, this study aimed to characterize the gastroprotective mechanisms of menthol (ME), the major compound of the essential oil from species of the genus Mentha. The gastroprotective action of ME was analyzed in gastric ulcers that were induced by ethanol or indomethacin in Wistar male rats. The mechanisms responsible for the gastroprotective effect were assessed by analyzing the amount of mucus secreted, involvement of non-protein sulfhydryl (NP-SH) compounds, involvement of calcium ion channels and NO/cGMP/K^+_{ATP} pathway, gastric antisecretory activity and the prostaglandin E_2 (PGE_2) production. The anti-diarrheal activity and acute toxicity of ME were also evaluated. Oral treatment with ME (50 mg/kg) offered 88.62% and 72.62% of gastroprotection against ethanol and indomethacin, respectively. There was an increased amount of mucus and PGE_2 production. The gastroprotective activity of ME involved NP-SH compounds and the stimulation of K^+_{ATP} channels, but not the activation of calcium ion channels or the production of NO. The oral administration of ME induced an antisecretory effect as it decreased the H^+ concentration in gastric juice. ME displayed anti-diarrheal and antiperistaltic activity. There were no signs of toxicity in the biochemical analyses performed in the rats' serum. These results demonstrated that ME provides gastroprotective and anti-diarrheal activities with no toxicity in rats.

1. Introduction

The gastric mucosa is continuously exposed to damaging agents that are involved in the pathogenesis of gastric ulcers. These damaging agents can be endogenous (e.g., hydrochloric acid, pepsin, refluxed bile, and reactive oxygen species) or exogenous (e.g., alcohol consumption, excessive coffee ingestion, and administration of non-steroidal anti-inflammatory drugs). The basic physiopathology of gastric ulcers results from an imbalance between damaging factors and cytoprotective factors, which include an intact mucus barrier, prostaglandins, adequate mucosal blood flow, activity of antioxidant compounds, and other mediators, although the exact ulcer etiology is complex and multifactorial [1].

Gastrointestinal diseases are major public health issues throughout the world and are estimated to affect 70% of the general population [2]. The oral administration of ethanol is widely used to induce experimental gastric ulcer because it is easily reproducible and rapidly penetrates into the gastric mucosa. Ethanol causes necrotic lesions of the gastric mucosa in a multifactorial way. Ethanol is known to induce ulcers in the glandular part of stomach due to the secretion of mast cell secretory products and reactive oxygen species [3]. Ethanol also acts by reducing the secretion of bicarbonate and the production of mucus, thus resulting in an increased flow of Na^+ and K^+, increased pepsin secretion, and a loss of H^+ ions into the lumen [4,5], which leads to cell necrosis and ulcer formation. HCl secretion further deepens necrosis and increases tissue injury. Because of these factors, ethanol-induced ulcers can be inhibited by agents that enhance mucosal defensive factors [6,7].

There is a continuous search for a new bioactive compound derived from medicinal plants and natural products with gastroprotective and ulcer-healing properties. The new compound should be accessible, safe, and gastroprotective, and it should effectively heal the ulcer, thus avoiding its recurrence [8]. Plants have presented promising results in the treatment of gastric ulcers in several research projects worldwide [9]. Among the natural compounds that have been studied in recent studies, several

* Corresponding author. Address: Morphology Department, Institute of Biosciences, UNESP, P.O. Box 510, 18618-970 Botucatu/SP, Brazil. Tel./fax: +55 014 3811 6264.
E-mail address: claudia@ibb.unesp.br (C.H. Pellizzon).

0009-2797/$ - see front matter © 2013 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.cbi.2013.10.003

terpenes have presented gastroprotective effects, such as limonene [10], suaveolol [11] and carvacrol [12]. Menthol (ME) is a cyclic terpene with molecular weight 156 kDa and the formula $C_{10}H_{20}O$. ME is the main compound of the essential oil from the species of the genus Mentha and is responsible for giving Mentha species their distinctive smell and flavor. Among the optical isomers, (−)−menthol occurs most widely in nature and is endowed with the peculiar property of being a fragrance and flavor compound [13]. This study aimed to characterize the mechanism of action of ME against ethanol- and indomethacin-induced gastric ulcers in rats. We also sought to evaluate the anti-diarrheal activity and the acute toxicity induced by ME.

2. Materials and methods

2.1. Menthol

(−)−Menthol (catalog #63660) was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Animals

Male Wistar rats (200–250 g) from the Central Animal House of UNESP were fed a certified diet with free access to tap water under standard light–dark cycles (12 h dark–12 h light), humidity (60 ± 1%) and temperature (21 ± 2 °C). All rats were fasted for at least 16 h prior to each experiment because the treatments were orally administered. Rats were housed in cages with raised, wide-mesh floors to prevent coprophagy. After each experiment, the rats were euthanized in a CO_2 chamber. All experimental protocols followed the recommendations of the Canadian Council on Animal Care and were approved by the UNESP Institutional Animal Care and Use Committee (permit number 221-CEEA, 2010).

2.3. Experimental assays

2.3.1. Ethanol-induced gastric ulcers: determination of dose

Male Wistar rats were distributed into five groups ($n = 7$) and then orally dosed with vehicle (10 mL/kg), carbenoxolone (100 mg/kg) or ME (25, 50 or 100 mg/kg). After 1 h, the animals received an oral dose of 1 mL of absolute ethanol. 1 h after ethanol treatment, the rats were euthanized, and their stomachs were removed [14]. The stomachs were then opened along the greater curvature and washed. The flattened stomach samples were scanned, and the ulcer area (mm^2) was measured using the AVSoft BioView software. The lower effective dose from the 3 doses tested was adopted for all other assays.

After scanning, stomach samples were collected for histological slide preparation and were either stained with hematoxylin and eosin (HE) or periodic acid-Schiff (PAS). A microscopic score [15] was determined for the following parameters: epithelial desquamation, hemorrhage, glandular damage, and eosinophilic infiltration. A scale ranging from 0 to 3 (0: none, 1: mild, 2: moderate, and 3: severe) was used for each criterion. The highest possible score was 12.

2.3.2. Involvement of the NO/cGMP/K^+ATP pathway in gastroprotection

Rats were distributed into eight groups ($n = 7$). Two groups of rats were subjected to intraperitoneal treatment with the following drugs: vehicle (8% tween 80, 10 mL/kg), L-NAME (N-nitro-L-arginine methyl ester 70 mg/kg, a NO synthase inhibitor), ODQ (1H[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one 10 mg/kg, a guanylate cyclase inhibitor) or glibenclamide (K^+_{ATP} channel blocker 3 mg/kg). 1 h later, the vehicle (10 mL/kg) and ME (50 mg/kg) were orally administered to four groups each [16]. After 60 min, all groups were orally treated with 1 mL of absolute ethanol for gastric ulcer induction. The rats were euthanized 1 h after ethanol administration, and the stomachs were removed, opened along the greater curvature, scanned and the ulcer area (mm^2) was determined using the AVSoft BioView software.

2.3.3. Involvement of non-protein sulfhydryl compounds (NP-SH) or calcium ion channels in gastroprotection

Rats were distributed into six groups ($n = 7$). Two groups of rats were subjected to intraperitoneal treatment with the following drugs: vehicle (8% tween 80, 10 mL/kg), NEM (N-ethylmaleimide 5 mg/kg, a NP-SH compounds blocker) or verapamil (a calcium channel blocker, 5 mg/kg). 1 h later, the vehicle (10 mL/kg) and ME (50 mg/kg) were orally administered to three groups each. After 60 min, all groups were orally treated with 1 mL of absolute ethanol for gastric ulcer induction. The rats were killed 1 h after ethanol administration, and the stomachs were removed, opened along the greater curvature, scanned and the ulcer area (mm^2) was determined using AVSoft BioView.

2.3.4. Evaluation of gastric juice parameters

Rats were randomly divided into six groups ($n = 7$). 30 min after oral treatment or immediately after the intra-duodenal administration of a single dose of vehicle (10 mL/kg), cimetidine (100 mg/kg) or ME (50 mg/kg), the rats were subjected to pyloric ligation [17]. 4 h later, the animals were euthanized, the abdomen was opened, and another ligature was placed around the esophagus, close to the diaphragm. The stomach was removed, and its contents were drained into a graduated centrifuge tube, which was then centrifuged at 2000*g* for 15 min. The total acid content of the gastric secretions was determined by titration to pH 7.0 with 0.01 N NaOH using a digital burette. The total concentration of acid was expressed as mEq/mL/4 h.

2.3.5. Determination of mucus adherence to the gastric wall

After 24 h of fasting, anesthetized rats ($n = 7$) were subjected to longitudinal incisions slightly below the xiphoid apophysis for the placement of a pyloric ligature. The oral administration of vehicle, carbenoxolone (200 mg/kg) or ME (50 mg/kg) was performed 1 h before the ligature. After 4 h, the rats were euthanized, and the glandular portion of the stomach was weighed, and immersed in Alcian Blue solution for the mucus quantification procedure. The absorbance was measured by ELISA in a spectrophotometer at a wavelength of 598 nm, and the results were expressed as μg Alcian Blue/g tissue [18].

2.3.6. Non-steroidal anti-inflammatory drug (NSAID)-induced gastric ulcers

Rats were distributed into three groups ($n = 7$). Vehicle (10 mL/kg), cimetidine (100 mg/kg) or ME (50 mg/kg) were orally administered 30 min prior to the induction of gastric lesions by the oral administration of the ulcerogenic agent indomethacin (100 mg/kg). The animals were euthanized 5 h after treatment with indomethacin [19]. The stomachs were removed, opened along the greater curvature and then scanned. The ulcer area (mm^2) was determined using AVSoft BioView.

2.3.7. Prostaglandin E_2 (PGE_2) assay

Rats were randomly divided into five groups ($n = 7$): sham, vehicle, vehicle + indomethacin, ME (50 mg/kg) and ME (50 mg/kg) + indomethacin. The +indomethacin groups subcutaneously received indomethacin (a non-steroidal anti-inflammatory drug, which inhibits the PGE_2 synthesis) at 30 mg/kg, and the other groups received vehicle. After 30 min, rats orally received vehicle or ME. The sham group received neither drug nor treatment. After

30 min, the rats were euthanized, and the stomachs were removed. Following stomach harvesting, the corpus was excised, weighed, and suspended in 10 mM PBS at pH 7.4 (1 mL). The tissue was minced finely with scissors and incubated at 37 °C for 20 min. After centrifugation at 9000*g*, the PGE_2 levels in the supernatant were measured by ELISA and read in spectrophotometer (420 nm) using a commercial kit (R&D Systems, Minneapolis, USA). The results were expressed as ng/mL [20].

2.3.8. Effect of menthol on castor oil-induced diarrhea

Three groups of rats were orally treated with vehicle (10 mL/kg), loperamide hydrochloride (3 mg/kg) or ME (50 mg/kg). After 30 min, each rat received 1 mL of castor oil orally. Immediately after ingesting the castor oil, each rat was kept in an individual cage, the floor of which was lined with blotting paper, and the rats were observed for 5 h. The following parameters were then observed: onset of diarrhea, number of solid, semi-solid, and watery feces, and total frequency of fecal outputs. Each rat received an evacuation index (EI) expressed according to the formula: EI = 1 × (number of solid stool) + 2 × (number of semi-solid stool) + 3 × (number of watery stool) [21].

2.3.9. Effect of menthol on gastrointestinal motility

Rats were orally treated with vehicle (10 mL/kg), loperamide hydrochloride (4 mg/kg) or ME (50 mg/kg). After 20 min, each rat orally received 1 mL of charcoal meal (10% charcoal suspension in 5% aqueous gum Arabic). After 30 min, rats were euthanized, and the stomach and small intestine were removed. The distance between the charcoal meal and the pylorus was measured and correlated to the distance from the pylorus to the caecum [22].

2.3.10. Acute toxicity

Male rats ($n = 10$) orally received vehicle or a single acute dose of ME (500 mg/kg, corresponding to ten times the therapeutic dose) after 12 h of fasting. Possible signs and symptoms associated with toxicity were observed at 0, 30, 60, 120, 180 and 240 min after the administration and then twice daily for the next 14 days. Body weights were noted daily. At the end of the period, the rats were euthanized, and the kidneys and liver were withdrawn, weighed and evaluated. Biochemical analyses were performed on the rats' serum to quantify AST (aspartate aminotransferase), ALT (alanine aminotransferase), γ-GT (gamma glutamyltransferase) and alkaline phosphatase to evaluate liver damage and creatinine and urea to evaluate kidney damage using the automated biochemical analyzer SBA-200, CELM, Brazil.

2.4. Statistical analysis

Parametric data were analyzed using an unpaired *t*-test or a one-way analysis of variance (ANOVA) followed by Dunnett's test and compared to the vehicle group or Tukey's test. The results were presented as the mean ± standard error of the mean (SEM). Nonparametric data (histology scoring) were analyzed using the Kruskal–Wallis (nonparametric ANOVA) test, followed by a Dunn multiple comparison test. The results were presented as the median (range). All analyses were performed using GraphPad InStat software. A value of $p < 0.05$ was considered significant.

3. Results

3.1. Ethanol-induced gastric ulcers

3.1.1. Gastric ulcer area

The vehicle group presented several hemorrhagic bands that were usually parallel to the long axis of the stomach, with an average ulcer area of 374.82 ± 12.75 mm^2. They were located mostly in the gastric corpus, and no visible lesions developed in the non-secretory part of the stomach. The two highest ME doses tested (i.e., 50 and 100 mg/kg) exhibited gastroprotective effects ($p < 0.01$) when compared to the vehicle group. ME presented a gastroprotective effect of 20.06% for the lower dose (25 mg/kg), 88.62% for 50 mg/kg (ulcer area 42.64 ± 15.64 mm^2) and 98.42% for the highest dose, 100 mg/kg (ulcer area 5.91 ± 5.33 mm^2). However, according to Tukey's test, there was no difference between the ulcer areas of the groups treated with 50 mg/kg or 100 mg/kg; therefore, the dose of 50 mg/kg was used for all subsequent experiments. The ulcer areas (mm^2) are represented in Fig. 1.

3.1.2. Microscopic analyses

Microscopically, ME presented moderate epithelial desquamation, mild hemorrhage, and glandular damage as well as an absence of eosinophilic infiltration, presenting a score of 3(1–3). Carbenoxolone showed moderate epithelial desquamation and glandular damage as well as mild hemorrhage and eosinophilic infiltration, presenting a score of 4(2–5). The score of the vehicle group was 12(11–12). The mucus polysaccharides were evidenced after the PAS staining, which conferred a purple barrier covering the gastric pits in the ME-treated rats. The HE and PAS staining of the ulcers are displayed in Fig. 2.

3.2. Involvement of the NO/cGMP/K^+_{ATP} pathway in gastroprotection

In rats that were pre-treated with L-NAME (a NO-synthase inhibitor) or ODQ (a guanylate cyclase inhibitor), the gastroprotective effect of ME (50 mg/kg) was maintained (62.65% and 97.71% gastroprotection, respectively, compared to the vehicle-treated group, $p < 0.01$), showing that the NO synthesis or cGMP were not involved in the protective mechanism of ME. However, in rats pretreated with glibenclamide (a K^+_{ATP} channel blocker), the gastroprotective effect of ME (50 mg/kg) was reversed, indicating the involvement of K^+_{ATP} channels in gastroprotection (Table 1).

3.3. Involvement of NP-SH compounds or calcium channels in gastroprotection

In rats pretreated with NEM (an NP-SH compound reagent), the gastroprotective effect of ME (50 mg/kg) was reversed, indicating the involvement of SH compounds in gastroprotection. However, in rats that were pre-treated with verapamil (a calcium ion channel blocker), the gastroprotective effect of ME (50 mg/kg) was maintained (a protective effect of 98.31% compared to the vehicle-treated group, $p < 0.001$), indicating that the calcium ion channels are not involved in the gastroprotective mechanism of ME (Table 2).

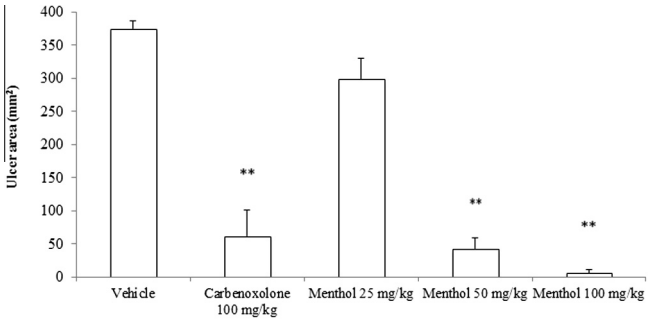

Fig. 1. Gastric ulcer area (mm^2) of rat stomachs with ethanol-induced gastric ulcers after treatment with vehicle, carbenoxolone (100 mg/kg) or menthol (25, 50 or 100 mg/kg). The results are reported as the mean ± SEM. ANOVA followed by Dunnett's test, $p < 0.01$.

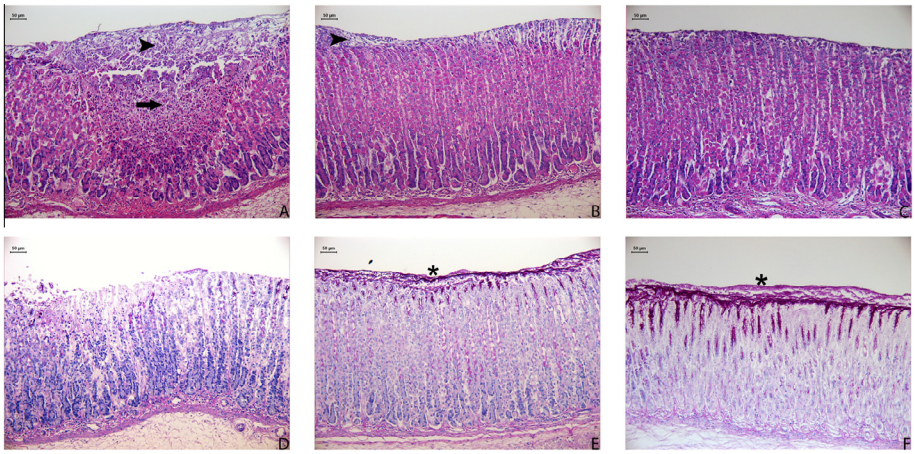

Fig. 2. Photomicrography of rat stomachs with ethanol-induced gastric ulcers after treatment with (A, D) vehicle, (B, E) carbenoxolone (100 mg/kg) or (C, F) menthol (50 mg/kg). In the HE staining (A, B, C), arrow head indicates epithelial desquamation and arrow indicates glandular damage. In the PAS staining (D, E, F), * indicates the mucus secretion in the gastric glands.

Table 1
Effect of menthol (50 mg/kg) on ethanol-induced gastric ulcer area (mm^2) in rats that were pretreated with L-NAME (NO synthase inhibitor), ODQ (guanylate cyclase inhibitor) or glibenclamide (K^+_{ATP} channels blocker).

Pretreatment (i.p)	Treatment (p.o)	Ulcer area (mm^2)	Gastroprotection (%)
Vehicle	Vehicle	501.70 ± 35.00	–
	Menthol	50.52 ± 10.99***	89.93
L-NAME 70 mg/kg	Vehicle	476.44 ± 89.17	–
	Menthol	177.94 ± 25.29**	62.65
ODQ 10 mg/kg	Vehicle	652.54 ± 144.21	–
	Menthol	14.94 ± 6.10**	97.71
Glibenclamide 3 mg/kg	Vehicle	134.13 ± 16.03	–
	Menthol	154.41 ± 28.50	0

The results are reported as the mean ± SEM. Unpaired *t* test, $^{**}p < 0.01$, $^{***}p < 0.001$.

Table 2
Effect of menthol (50 mg/kg) on ethanol-induced gastric ulcer area (mm^2) in rats that were pretreated with NEM (SH blocker) or verapamil (calcium channel blocker).

Pretreatment (i.p)	Treatment (p.o)	Ulcer area (mm^2)	Gastroprotection (%)
Vehicle	Vehicle	1825.62 ± 192.76	–
	Menthol	17.86 ± 11.46***	99.02
NEM 5 mg/kg	Vehicle	1799.24 ± 250.73	–
	Menthol	1574.03 ± 346.59	8.34
Verapamil 5 mg/kg	Vehicle	1155.17 ± 336.88	–
	Menthol	19.53 ± 12.38**	98.31

The results are reported as the mean ± SEM. Unpaired *t* test, $^{**}p < 0.01$, $^{***}p < 0.001$.

Table 3
Effect of menthol (50 mg/kg) on gastric juice parameters in rats with pyloric ligation.

Route	Treatment	Gastric juice volume (mL)	$[H^+]$ mequiv/mL/4 h
Oral	Vehicle	2.93 ± 0.36	9.60 ± 0.95
	cimetidine 100 mg/kg	1.9 ± 0.13	2.23 ± 0.18**
	Menthol 50 mg/kg	3.39 ± 0.63	5.90 ± 0.67**
Intraduodenal	Vehicle	5.08 ± 0.21	10.23 ± 0.44
	Cimetidine 100 mg/kg	2.42 ± 0.17**	4.62 ± 0.50**
	Menthol 50 mg/kg	2.95 ± 0.15**	9.05 ± 0.32

The results are reported as the mean ± SEM. ANOVA followed by Dunnett's test, $p < 0.01$.

3.4. Evaluation of the gastric juice parameters

A comparison of the gastric juice parameters of rats treated with oral or intra-duodenal ME (50 mg/kg) demonstrated that the oral treatment was able to diminish the H^+ concentration ($p < 0.01$) in the gastric juice without modifying its volume. The intra-duodenal administration was not able to decrease the H^+ concentration, but it did diminish the volume of the gastric juice (Table 3).

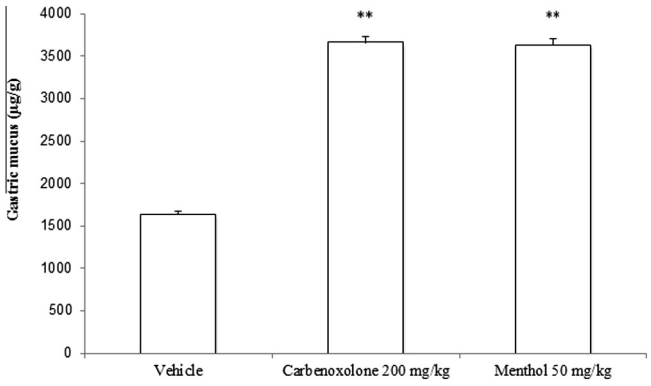

Fig. 3. Quantification of adherent mucus (µg/g of tissue) in the gastric mucosa of rats treated with vehicle, carbenoxolone (200 mg/kg) or menthol (50 mg/kg). The results are reported as the mean ± SEM. ANOVA followed by Dunnett's test, $p < 0.01$.

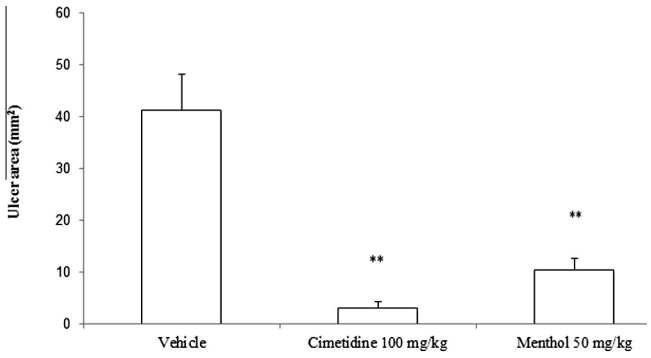

Fig. 4. Gastric ulcer area (mm^2) in rat stomachs with indomethacin-induced gastric ulcers after treatment with vehicle, carbenoxolone (100 mg/kg) or menthol (50 mg/kg). The results are reported as the mean ± SEM. ANOVA followed by Dunnett's test, compared to vehicle group, $p < 0.01$.

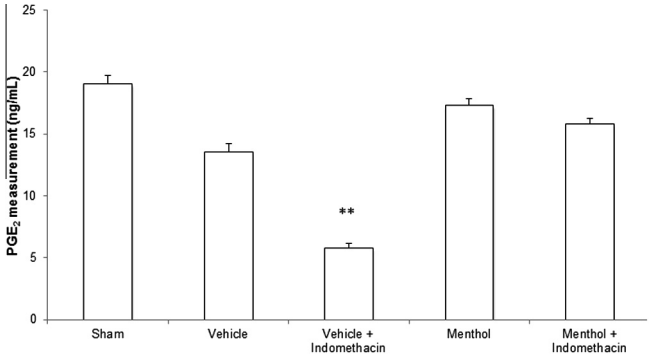

Fig. 5. PGE_2 measurement (ng/mL) in the gastric mucosa of rats treated with vehicle or menthol (50 mg/kg) alone or in association with nonselective COX inhibitor (indomethacin). The results are reported as the mean ± SEM. ANOVA followed by Dunnett's test, compared to sham group, $p < 0.01$.

3.5. Determination of mucus adherence to the gastric wall

There was a 2.2-fold increase in the amount of gastric mucus adhering to the stomach wall in the ME-treated (50 mg/kg) group (3632.00 ± 66.06 µg/g, $p < 0.01$) compared to the vehicle-treated group (1633.43 ± 43.73 µg/g) (Fig. 3).

3.6. Indomethacin-induced gastric ulcers

The vehicle-treated group had a large quantity of small petechiae in the stomach and a mean ulcer area of 38.06 ± 8.02 mm^2. In this model, ME presented a gastroprotective effect of 72.62% (ulcer area 10.42 ± 2.71 mm^2, $p < 0.01$) in comparison to the vehicle group (Fig. 4).

Table 4
Evacuation index and % of evacuation inhibition after oral treatment with vehicle, loperamide hydrochloride (3 mg/kg) or menthol (50 mg/kg).

Treatment	Evacuation index	% of evacuation inhibition
Vehicle	13.0 ± 0.84	–
Loperamide 3 mg/kg	8.0 ± 1.0**	38.50
Menthol 50 mg/kg	8.3 ± 0.42**	36.15

The results are reported as the mean ± SEM. ANOVA followed by Dunnett's test, $p < 0.01$.

Table 5
Evaluation of intestinal motility ($cm^{1/2}$) after oral treatment with vehicle, loperamide hydrochloride (5 mg/kg) or menthol (50 mg/kg).

Treatment	Intestinal motility	% of motility inhibition
Vehicle	4.15 ± 0.31	–
Loperamide 5 mg/kg	1.91 ± 0.23**	53.97
Menthol 50 mg/kg	1.81 ± 0.32**	56.38

The results are reported as the mean ± SEM. ANOVA followed by Dunnett's test, $p < 0.01$.

3.7. PGE_2 assay

The decreased level ($p < 0.01$) of PGE_2 in the vehicle + indomethacin group, compared to that of the sham group, proves that indomethacin induces decrease of PGE_2 production. The ME groups maintained PGE_2 levels near that of the sham group, even with indomethacin administration (Fig. 5).

3.8. Effect of menthol on castor oil-induced diarrhea and gastrointestinal motility

ME inhibited evacuation in 36.15% of the treated group compared to the vehicle group ($p < 0.01$), indicating an antidiarrheal activity comparable to the effect of the standard drug loperamide hydrochloride (38.50%, Table 4). The antidiarrheal effect of ME was connected to an antiperistaltic activity, decreasing the intestinal motility in 56.18% of the treated animals compared to the vehicle group ($p < 0.01$, Table 5).

Table 6
Effect of orally administered menthol (500 mg/kg) on rats' body weight, organ weight, and serum biochemical parameters.

	Vehicle	Menthol
Initial body weight (day 0)	196.70 ± 5.31	190.45 ± 4.54
Final body weight (day 14)	290.60 ± 8.29	272.67 ± 8.11
Kidneys weight	0.15 ± 0.00	0.15 ± 0.00
Liver weight	0.72 ± 0.02	0.70 ± 0.02
AST	105.80 ± 6.24	115.70 ± 7.44
ALT	46.90 ± 0.99	47.90 ± 1.00
Gama-GT	5.36 ± 0.46	5.09 ± 0.58
Alkaline phosphatase	295.00 ± 10.71	277.37 ± 14.30
Creatinine	0.28 ± 0.02	0.30 ± 0.03
Urea	45.30 ± 1.51	42.44 ± 1.20

Body weights are expressed in g. Organ weights are expressed in comparison to the body weight ($g^{1/2}$). AST, ALT, γ-GT and alkaline phosphatase are expressed in U/L, creatinine and urea are expressed in mg/dL. The results are reported as the mean ± SEM. Unpaired t test.

3.9. Acute toxicity

No signs or symptoms associated with toxicity were observed on the day of oral administration or over the subsequent 14 days. Body weights progressed normally. After the rats were euthanized, there were no alterations in the macroscopic appearance of the kidneys or liver or in their weights. There were no alterations in the biochemical parameters analyzed in the rats' serum between the groups, indicating that there was no hepatotoxicity or nephrotoxicity associated with the ME oral treatment (Table 6).

4. Discussion

The present study investigated the gastroprotective activity of (−)−menthol (ME) in experimental models of gastric ulcer and its possible mechanisms of action. In the ethanol-induced gastric ulcer model, ME exhibited a dose-dependent effect and exerted substantial protective action on the gastric mucosa. The lowest effective dose (50 mg/kg) was used for all subsequent experiments. Considering the multifactorial ways in which ethanol exerts its ulcerogenic action, the possible mechanisms of gastroprotection conferred by ME were investigated as follows.

The cellular perturbation caused by a damaging agent such as ethanol disrupts the normal Ca2+ homeostasis. Ethanol administration leads to an intracellular Ca2+ accumulation, providing an injurious action in the gastric mucosa [23,24]. The oral administration of the calcium ion channel blocker verapamil was not able to reverse the ME gastroprotective effect, indicating that the blockade of the calcium ions efflux did not interfere with the protective mechanism, and therefore, that this gastroprotective pathway is not involved in ME activity.

Several studies have shown the importance of mucus secretion in gastroprotection [8,25]. The mucus barrier is considered the first line of mucosal defense because it decreases physical damage to the epithelium by ingested foods. It is an important barrier against self-digestion [26] and acts as an antioxidant, scavenging free radicals in the mucosa [27]. Ulcerogenic substances cause disruptions in this barrier and allow contact between the gastric juice and epithelial cells, leading to mucosal injury [28]. Thus, the increased stimulation of mucus production by 2.2-fold over the control group is a relevant part of the hypothesized mechanism of gastric mucosal protection by ME. The increase in mucus secretion was easily evidenced in the photomicrographies of the PAS-stained gastric ulcer.

Endogenous NP-SH compounds help maintain the integrity of the mucus barrier by uniting its subunits by disulfide bridges, preventing the mucus from becoming soluble, and easily withdrawn by ulcerogenic agents, including ethanol [29]. NP-SH compounds also prevent the production of free radicals by ethanol and act as recycling antioxidants [30]. The rats that were pretreated with an inhibitor of NP-SH compounds presented ulcer areas similar to those of the vehicle-treated rats, indicating the importance of an intact NP-SH barrier to the maintenance of the ME gastroprotective effect.

Mucus secretion also can be regulated by nitric oxide (NO) [31], an endogenous gaseous mediator synthesized by the enzyme NO-synthase (NOs). NO also plays key roles in enhancing blood flow [32], regulating acid secretion and inhibiting neutrophil aggregation [33] and leukocyte adherence to the vascular endothelium [34]. Our results showed that despite the inhibition of NO synthesis by the action of the NO-synthase inhibitor (L-NAME), ME exerted a gastroprotection similar to that observed in the group receiving ME treatment without NO blocking, indicating that maintaining NO synthesis is not crucial to gastroprotection by ME.

In the gastric mucosa, NO interacts with neuropeptides and prostaglandins to maintain mucosal integrity. NO activates guanylyl cyclase to increase cyclic guanosine monophosphate (cGMP) levels and subsequently activates the ATP sensitive potassium channels (K^+_{ATP}). The activation of this NO/cGMP/K^+_{ATP} pathway leads to gastroprotection [35]. K^+_{ATP} channels mediate gastroprotection by enhancing gastric microcirculation and inhibiting neutrophil activation and the subsequent superoxide production [36]. Despite this mediation, there was no loss of gastroprotection after the inhibition of NO synthesis or the cGMP blockade, but the blockade of the K^+_{ATP} channels reversed the gastroprotective action of ME, thus indicating the involvement of these channels in the mechanism of action of ME.

K^+_{ATP} channels are not exclusively activated by the NO pathway but can also be activated by PGE_2 activity [37]. The oral treatment with ME was able to maintain PGE_2 levels, even after the administration of the COX-inhibitor indomethacin, indicating the importance of PGE_2 in the mechanism of action of ME. This result can explain the activation of the K^+_{ATP} channels as well as the cytoprotective activity observed in the indomethacin-induced gastric ulcer. Indomethacin, as a non-steroidal anti-inflammatory drug (NSAID), induces gastric damage mainly by inhibiting prostaglandin production through inhibiting the activity of COX-1 and COX-2 isozymes [38,39].

The physiological functions of PGE_2 in the gastrointestinal tract include stimulating bicarbonate and mucus secretion, maintaining mucosal integrity, and inducing a trophic effect in gastric and intestinal mucosa by triggering mitogenic signaling in mucosal cells [40]. It is also known that PGE_2 presents a strong anti-secretory activity [41], which can, at least in part, explain the anti-secretory effect observed after the oral administration of ME. The H^+ concentration was decreased, but the gastric juice volume was not altered. This effect did not occur in the intra-duodenally treated rats, leading to the conclusion that ME acts via a local rather than a systemic mechanism. In addition to the increasing effect on PGE_2 production, this antisecretory effect may also be due to increased mucus secretion, as the mucus barrier is able to neutralize secreted H^+.

This study also evaluated ME activity in castor oil-induced diarrhea and gastrointestinal motility. Experimentally, castor oil induces diarrhea by increasing the secretion of fluids and electrolytes in the intestinal lumen, thus resulting in fluid accumulation and in an aqueous content that flows rapidly in the small and large intestines [42]. In this assay, ME displayed significant activity against the diarrhea induced by castor oil, an effect that can be compared to the standard drug loperamide. The antidiarrheal effect of ME was accompanied by an antiperistaltic effect. This is an important pathway of gastroprotection, since the incidence of gastric ulcers can be influenced by decreasing in gastric motility. The relaxation of circular muscles in the stomach avoid the incidence of ulcers through inducing the flattening of the folds, which leads to an increase in the mucosal area exposed to the necrotizing agent and reduce the volume of the agent on the rugal crest [25].

The acute toxicity test did not show any signs of toxicity or mortality. Behavioral changes such as irritation, restlessness, respiratory distress, abnormal locomotion, and catalepsy were not observed over a period of 14 days. There were no alterations in the dosage of renal and hepatic enzymes or in body weight evolution, revealing that ME has no toxicity when administered orally at a dose of 500 mg/kg, which is ten times higher than the therapeutic dose employed in this study.

5. Conclusion

The results described here suggest that menthol presents anti-ulcer activities against ethanol and indomethacin. The gastroprotective activity of menthol is associated mainly with mucus secretion, which is related to the maintenance of NP-SH

compounds, PGE_2 production and K^+_{ATP} channel activation and to an anti-secretory effect. Connected to the gastroprotective effect, menthol also presents an antidiarrheal and antiperistaltic effect. No signs of acute toxicity have been associated with the administration of a high dose of menthol. However, further clinical and toxicological studies must be conducted to support the use of menthol as a potential antiulcerogenic drug.

6. Conflict of interest

There are no conflicts of interest to disclose.

Acknowledgments

This research was supported by FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo, 10/08536-9) and CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior). The authors want to thank V.M. Souza, F.J. Fontana and M.R.R. Sarzi for technical support (Laboratório de Histologia do Departamento de Clínica Médica, FMB/UNESP).

References

[1] L. Laine, K. Takeuchi, A. Tarnawski, Gastric mucosal defense and cytoprotection: bench to bedside, Gastroenterology 135 (2008) 41–60.
[2] H. Ouyang, J.D. Chen, Review article: therapeutic roles of acupuncture in functional gastrointestinal disorders, Aliment. Pharmacol. Ther. 20 (2004) 831–841.
[3] C.V. Rao, S.K. Ojha, K. Radhakrishnan, et al., Antiulcer of *Utleria salicifolia* rhizome extract, J. Ethnopharmacol. 91 (2004) 243–249.
[4] S. Szabo, Mechanisms of mucosal injury in the stomach and duodenum: time-sequence analysis of morphologic, functional, biochemical and histochemical studies, Scand. J. Gastroenterol. 22 (1987) 21–28.
[5] A. Mahmood, K. Sidik, I. Salmah, K. Suzainor, K. Philip, Antiulcerogenic activity of *Ageratum conyzoides* leaf extract against ethanol-induced gastric ulcer in rats as animal model, Int. J. Mol. Med. 1 (2005) 402–405.
[6] Y. Morimoto, K. Shimohara, S. Oshima, T. Sukamoto, Effects of the new antiulcer agent KB-5492 on experimental gastric mucosal lesions and gastric mucosal defensive factors, as compared to those of teprenone and cimetidine, Jpn. J. Pharmacol. 57 (1991) 495–505.
[7] G. Sener, K. Paskaloglu, G. Anyaanoglu-Dulger, Protective effect of increasing doses of famotidine, omeprazole, lansoprazole, and melatonin against ethanol-induced gastric damage in rats, Indian J. Pharmacol. 36 (2004) 171–174.
[8] D. Laloo, S.K. Prasad, S. Krishnamurthy, S. Hemalatha, Gastroprotective activity of ethanolic root extract of *Potentilla fulgens* Wall. ex Hook, J. Ethnopharmacol. 146 (2013) 505–514.
[9] G. Schmeda-Hirschmann, E. Yesilada, Traditional medicine and gastroprotective crude drugs, J. Ethnopharmacol. 100 (2005) 61–66.
[10] A.L. Rozza, T.M. Moraes, H. Kushima, et al., Gastroprotective mechanisms of Citrus lemon (Rutaceae) essential oil and its majority compounds limonene and β-pinene: involvement of heat-shock protein-70, vasoactive intestinal peptide, glutathione, sulfhydryl compounds, nitric oxide and prostaglandin E_2, Chem. Biol. Interact. 189 (2011) 82–89.
[11] C. Vera-Arzave, L.C. Antonio, J. Arrieta, et al., Gastroprotection of suaveolol, isolated from *Hyptis suaveolens*, against ethanol-induced gastric lesions in Wistar rats: role of prostaglandins, nitric oxide and sulfhydryls, Molecules 17 (2012) 8917–8927.
[12] F.V. Silva, A.G. Guimarães, E.R. Silva, et al., Anti-inflammatory and anti-ulcer activities of carvacrol, a monoterpene present in the essential oil of oregano, J. Med. Food 15 (2012) 984–991.
[13] R. Eccles, Menthol and related cooling compounds, J. Pharm. Pharmacol. 46 (1994) 18–630.
[14] A. Robert, J.E. Nezamis, C. Lancaster, A.J. Hanchar, Cytoprotection by prostaglandins in rats. Prevention of gastric necrosis produced by alcohol, HCl, NaOH, hypertonic NaCl, and thermal injury, Gastroenterology 77 (1979) 433–443.
[15] N. Jahovic, G. Erkanli, S. Işeri, S. Arbak, I. Alican, Gastric protection by alpha-melanocyte-stimulating hormone against ethanol in rats: involvement of somatostatin, Life Sci. 80 (2007) 1040–1045.
[16] A.E. Chávez-Piña, G.R. Tapia-Álvarez, A. Reyes-Ramínrez, A. Navarrete, Carbenoxolone gastroprotective mechanism: participation of nitric oxide/(c)GMP/K(ATP) pathway in ethanol-induced gastric injury in the rat, Fund. Clin. Pharmacol. 25 (2011) 717–722.
[17] H. Shay, S.A. Komarov, S.S. Fels, et al., A simple method for the uniform production of gastric ulceration in the rat, Gastroenterology 5 (1945) 43–61.
[18] S. Rafatullah, M. Tariq, M.A. Al-Yahya, J.S. Mossa, A.M. Ageel, Evaluation of turmeric (*Curcuma longa*) for gastric and duodenal antiulcer activity in rats, J. Ethnopharmacol. 29 (1990) 25–34.
[19] I. Puscas, C. Puscas, M. Coltau, et al., Comparative study of the safety and efficacy of ebrotidine versus ranitidine and placebo in the prevention of piroxicam-induced gastroduodenal lesions, Arzneim.-Forsch. 47 (1997) 568–572.
[20] G.H. Curtis, W.K. Macnaughton, D.G. Gall, J.L. Wallace, Intraluminal pH modulates gastric prostaglandin synthesis, Can. J. Physiol. Pharmacol. 73 (1995) 130–134.
[21] T. Crocci, X. Emonds-Alt, L. Manara, SR 48968 selectively prevents faecal excretion following activation of tachykinin NK2 receptors in rats, J. Pharm. Pharmacol. 46 (1994) 383–385.
[22] F. Calzada, R. Arista, H. Pérez, Effect of plants used in Mexico to treat gastrointestinal disorders on charcoal–gum acacia-induced hyperperistalsis in rats, J. Ethnopharmacol. 128 (2010) 49–51.
[23] E.R. Kokoska, G.S. Smith, A.B. Wolff, et al., Role of calcium in adaptive cytoprotection and cell injury induced by deoxycholate in human gastric cells, Am. J. Physiol. Gastrointest. Liver Physiol. 275 (1998) G322–G330.
[24] T.A. Miller, E.R. Kokoska, G.S. Smith, A. Banan, Role of calcium homeostasis in gastric mucosal injury and protection, Life Sci. 69 (2001) 3091–3102.
[25] A.S. AlRashdi, S.M. Salama, S.S. Alkiyumi, et al., Mechanisms of gastroprotective effects of ethanolic leaf extract of *Jasminum sambac* against HCl/ethanol-induced gastric mucosal injury in rats, Evid. Based Complement. Alternat. Med. (2012). article ID 786426.
[26] J.L. Wallace, Prostaglandins, NSAIDs, and gastric mucosal protection: why doesn't the stomach digest itself?, Physiol Rev. 88 (2008) 1547–1565.
[27] M.G. Repetto, S.F. Llesuy, Antioxidant properties of natural compounds used in popular medicine for gastric ulcers, Braz. J. Med. Biol. Res. 35 (2002) 523–534.
[28] R.L. Darling, J.J. Romero, E.J. Dial, J.K. Akunda, R. Langenbach, L.M. Lichtenberger, The effects of aspirin on gastric mucosal integrity, surface hydrophobic, and prostaglandin metabolism in cyclooxygenase knockout mice, Gastroenterology 127 (2004) 94–104.
[29] J.R. Avila, C. Alarcón De La Lastra, M.J. Martín, et al., Role of endogenous sulphydryls and neutrophil infiltration in the pathogenesis of gastric mucosal injury induced by piroxicam in rats, Inflamm. Res. 45 (1996) 83–88.
[30] D. Banerjee, B. Maity, S.K. Nag, S.K. Bandyopadhyay, S. Chattopadhyay, Healing potential of *Picrorhiza kurroa* (Scrofulariaceae) rhizomes against indomethacin-induced gastric ulceration: a mechanistic exploration, BMC Complement. Altern. Med. 8 (2008) 3.
[31] J.F. Brown, A.C. Keates, P.J. Hanson, B.J. Whittle, Nitric oxide generators and cGMP stimulate mucus secretion by rat gastric mucosal cells, Am. J. Physiol. Gastrointest. Liver Physiol. 265 (1993) 418–422.
[32] J.L. Wallace, M.J. Miller, Nitric oxide in mucosal defense: a little goes a longway, Gastroenterology 119 (2000) 512–520.
[33] J.L. Wallace, W. McKnight, T.L. Wilson, P. Del Soldato, G. Cirino, Reduction of shock-induced gastric damage by a nitric oxide-releasing aspirin derivative: role of neutrophils, Am. J. Physiol. Gastrointest. Liver Physiol. 273 (1997) 1246–1251.
[34] R.C. Zanardo, V. Brancaleone, E. Distrutti, S. Fiorucci, G. Cirino, J.L. Wallace, Hydrogen sulfide is an endogenous modulator of leukocyte-mediated inflammation, FASEB J. 20 (2006) 2118–2120.
[35] J.V. Medeiros, G.G. Gadelha, S.J. Lima, et al., Role of the NO/cGMP/K(ATP) pathway in the protective effects of sildenafil against ethanol-induced gastric damage in rats, Br. J. Pharmacol. 153 (2008) 721–727.
[36] D.A. Campos, A.F. de Lima, S.R. Ribeiro, et al., Gastroprotective effect of a flavone from *Lonchocarpus araripensis* Benth. (Leguminosae) and the possible mechanism, J. Pharm. Pharmacol. 60 (2008) 391–397.
[37] S.R. Lira, V.S. Rao, A.C. Carvalho, et al., Gastroprotective effect of lupeol on ethanol-induced gastric damage and the underlying mechanism, Inflammopharmacology 17 (2009) 221–228.
[38] J.L. Wallace, W. McKnight, B.K. Reuter, N. Vergnolle, NSAID-induced gastric damage in rats: requirement for inhibition of both cyclooxygenase 1 and 2, Gastroenterology (2000) 706–714.
[39] K. Takeuchi, A. Tanaka, S. Kato, K. Amagase, H. Satoh, Roles of COX inhibition in pathogenesis of NSAID-induced small intestinal damage, Clin. Chim. Acta 411 (2010) 459–466.
[40] R. Pai, B. Soreghan, I.L. Szabo, M. Pavelka, D. Baatar, A.S. Tarnawski, Prostaglandin E_2 transactivates EGF receptor: a novel mechanism for promoting colon cancer growth and gastrointestinal hypertrophy, Nat. Med. 8 (2002) 289–293.
[41] B.M. Peskar, N. Maricic, Role of prostaglandins in gastroprotection, Dig. Dis. Sci. 43 (1998) 23S–29S.
[42] T.F. Burks, Gastrointestinal drugs, in: K. Kist (Ed.), Human Pharmacology: Molecular to Clinical, Wolfe Publishing Ltd., London, UK, 1991, pp. 789–800.